# Nuclear receptors in control of cholesterol transport

van der Veen, Jelske Nynke

# CHAPTER 5

## Abcg5/Abcg8-independent pathways contribute to hepatobiliary cholesterol secretion in mice

T.Plösch[*], J.N. van der Veen[*], R. Havinga, N.C.A. Huijkman, V.W. Bloks, F. Kuipers

Center for Liver, Digestive, and Metabolic Diseases, Department of Pediatrics, University Medical Center Groningen, Groningen, The Netherlands

[*]Both authors contributed equally to this work.

Am J Physiol Gastrointest Liver Physiol 2006;291: G414-423

## ABSTRACT

The ABC half-transporters ABCG5 and ABCG8 heterodimerize into a functional complex that mediates secretion of plant sterols and cholesterol by hepatocytes into bile and their apical efflux from enterocytes. We have addressed the putative rate-controlling role of Abcg5/Abcg8 in hepatobiliary cholesterol excretion in mice during (maximal) stimulation of this process. Despite similar bile salt (BS) excretion rates, basal total sterol and phospholipid (PL) output rates were reduced by 82 % and 35 %, respectively, in chow-fed *Abcg5*$^{-/-}$ mice compared to wild-type mice. Upon infusion with the hydrophilic bile salt TUDCA, similar relative increases in bile flow, BS output, PL output and total sterol output were observed in wild-type, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice. Maximal cholesterol and PL output rates in *Abcg5*$^{-/-}$ mice were only 15 % and 69 %, respectively, of wild-type values. Infusion of increasing amounts of the hydrophobic bile salt TDCA increased cholesterol excretion 3.0- and 2.4-fold in wild-type and *Abcg5*$^{-/-}$ mice, but rapidly induced cholestasis in *Abcg5*$^{-/-}$ mice. Treatment with the LXR agonist T0901317 increased the maximal sterol excretion capacity in wild-type mice (4-fold), concomitant with induction of *Abcg5*/*Abcg8* expression, but not in *Abcg5*$^{-/-}$ mice. In a separate study, mice were fed chow containing 1 % w/w cholesterol. As expected, hepatic expression of *Abcg5* and *Abcg8* was strongly induced (5-fold and 4-fold) in wild-type but not in liver x-receptor α-deficient (*Lxra*$^{-/-}$) mice. Surprisingly, hepatobiliary cholesterol excretion was increased to the same extent, i.e., 2.2-fold in wild-type mice and 2.0-fold in *Lxra*$^{-/-}$ mice, upon cholesterol-feeding. Our data confirm that Abcg5, as part of the Abcg5/Abcg8 heterodimer, strongly controls hepatobiliary cholesterol secretion in mice. However, our data demonstrate that Abcg5/Abcg8 heterodimer-independent, inducible routes exist that can significantly contribute to total hepatobiliary cholesterol output.

# INTRODUCTION

Mutations in the ATP-binding-cassette (ABC) half-transporters ABCG5 and ABCG8 cause sitosterolemia[1,2] which is characterized by the accumulation of plant sterols in the body[3]. Data indicate that ABCG5 and ABCG8, which are highly expressed in liver and small intestine, heterodimerize into a functional complex[1,4]. Mutations in either one of the genes cause the biochemical hallmarks of the disease in humans[1,2] as well as in mouse models[5,6]. The daily intake of plant sterols, i.e., sitosterol and campesterol, from a "Western-type" diet is in the same order of magnitude as that of cholesterol. However, only trace amount of plant sterols are absorbed in healthy subjects[7,8]. ABCG5/ABCG8 mediates efflux of plant sterols from enterocytes back into the intestinal lumen and their excretion into bile, thus limiting their accumulation in the body[1,9]. Expression of the *Abcg5* and *Abcg8* genes is controlled by the liver X-receptor (LXRα/NR1H3) and possibly by the liver-receptor-homologue 1 (LRH-1)[10,11]. LXR is activated by oxysterols and hence is considered a cellular "cholesterol sensor"[12].

Kosters *et al.*[13] have demonstrated that, across various mouse models, a strong correlation exists between biliary cholesterol excretion (normalized for bile salt and phospholipid excretion) and hepatic *Abcg5/g8* expression[13]. Overexpression of *Abcg5/Abcg8* in transgenic mice[14] or induction of their expression *via* activation of LXR with synthetic ligands[6,11] or dietary cholesterol feeding[15,16] in wild-type mice is associated with a strongly increased hepatobiliary cholesterol excretion. Deletion of the genes, either *Abcg5*[6] or *Abcg8*[5] or both[17], in general has the opposite effects. Importantly, biliary cholesterol content is also reduced in heterozygote *Abcg5*$^{+/-}$, *Abcg8*$^{+/-}$ and *Abcg5*$^{+/-}$/*Abcg8*$^{+/-}$ mice[5,6,17], indicating a high degree of control of the functional heterodimer in the secretion process. However, it is of importance to note that residual cholesterol secretion is still observed in the complete knock-out mice (10-20% of wild-type[16,17]). In addition, it appeared that diosgenin-induced hypersecretion of cholesterol in mice occurs in the absence of Abcg5/Abcg8 induction[18] although the presence of functional Abcg5/Abcg8 is required for the effect to occur[16]. Of note, in most of the studies mentioned, cholesterol content of gallbladder bile was determined rather than biliary output rates. In contrast to the mouse[13], recent data demonstrate that in human liver transplantation patients no relationship exists between normalized biliary cholesterol excretion and hepatic *ABCG5* and *ABCG8* gene expression[19].

Thus it appears likely to assume that hepatobiliary cholesterol secretion can occur by both Abcg5/g8-dependent and Abcg5/g8 independent routes, the former being (quantitatively) the most important one in mice under basal conditions. In this study, we have used two strategies to address the quantitative contribution of both (putative) pathways under stressed conditions, *i.e.*, during infusion of hydrophilic and hydrophobic bile salts in control and LXR-agonist-treated wild-type, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice and upon feeding a high-cholesterol diet in wild-type and *Lxra*$^{-/-}$ mice. The data from these studies provide evidence to suggest that, under certain metabolic conditions, Abcg5/Abcg8-independent routes significantly contribute to total hepatobiliary cholesterol output in mice.

# MATERIALS AND METHODS

### *Animals and diets.*

Mice homozygous (*Abcg5*$^{-/-}$) and heterozygous (*Abcg5*$^{+/-}$) for the disruption of the *Abcg5* gene and their wild-type littermates were used[6]. Animals were housed in temperature-controlled rooms (21°C) with 12 hours light cycling and received standard mouse chow (Arie Blok, Woerden, The Netherlands) and water *ad libitum*. The diet contained 0.017 % (w/w) cholesterol and 0.045 % (w/w) plant sterols.

*Lxra*$^{-/-}$ mice, generated by Deltagen, Inc. (Redwood City, USA) using standard gene-targeting methods, were kindly provided by Tularik Inc. (South San Francisco, USA). In short, a 42 bp fragment corresponding to a segment of exon 2 was replaced by a beta-galactosidase cDNA and a phosphoglycerate kinase promoter-driven neomycin resistance cassette. The remaining procedure followed that described for the *Abcg5*$^{-/-}$ mice[6]. Mice were genotyped via PCR using allele-specific primers (wild-type: GTTTCTCTCCCCTATCTA-TCTAGGGAGAC; CACCCATTCTCCCGTGCTTCTCTTG; knockout: GGGCCAGCTCATTC-CTCCCACTCAT). Mice homozygous (*Lxra*$^{-/-}$) and heterozygous (*Lxra*$^{+/-}$) for the disruption of the *Lxra* gene and their wild-type littermates received either standard mouse chow or chow diet containing 1 % cholesterol (wt/wt; Arie Blok, Woerden, The Netherlands) for two weeks. Male mice of 2-4 months were used. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

***Abcg5*$^{-/-}$ *mice.*** *Abcg5*$^{-/-}$ and wild-type mice were fed either standard laboratory chow or chow supplemented with the synthetic LXR-agonist T0901317 (0.015 %, w/w; Cayman Chemicals, Ann Arbor, MI, USA) for 7 days. *Abcg5*$^{+/-}$ mice received standard chow only. Female mice of 3-6 months were used.

Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder. After two basal bile samples of 15 minutes, mice were continuously infused with tauroursodeoxycholate (TUDCA; Calbiochem/Merck Biosciences, Darmstadt, Germany) or taurodeoxycholate (TDCA; Calbiochem/Merck Biosciences) in PBS via the jugular vein. Infusion rates were increased in a stepwise manner: 150, 300, 450, 600 nmol/min and 25, 50, 75, 100 nmol/min for TUDCA and TDCA, respectively. During bile collection, body temperature was stabilized using an humidified incubator. At the end of the collection period, animals were killed by cardiac puncture. Livers were excised and weighed.

Gene expression, plasma lipid composition and hepatic lipid contents was studied in an independent experiment. 5-7 months old male *Abcg5*$^{-/-}$, *Abcg5*$^{+/-}$ and wild-type mice were fed standard laboratory chow. Mice were anesthetized with isoflurane and killed by cardiac puncture. Blood was collected in EDTA-containing tubes. Livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses and RNA isolation.

***Lxra*$^{-/-}$ *mice.*** *Lxra*$^{-/-}$, *Lxra*$^{+/-}$ and *Lxra*$^{+/+}$ mice were anaesthetized by intraperitoneal injection with Hypnorm and diazepam as described above. Bile was collected by cannulation of the gallbladder for 30 minutes. Body temperature was stabilized using an humidified incubator. Subsequently, animals were killed by cardiac puncture and livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses and RNA isolation. TUDCA infusion in *Lxra*$^{-/-}$ mice and wild-type littermates fed a high-cholesterol diet was performed as described above.

### *Analytical procedures.*

Biliary bile salt concentrations were measured enzymatically[20]. Biliary phospholipid and sterol concentrations in the Abcg5 experiments were determined as described previously[21]. No distinction was made between cholesterol and plant sterols, as enzymatic cholesterol assays have been found to measure both[22]. In *Lxra* mice, phospholipids and cholesterol in bile were determined as described by Böttcher *et al.*[23] and Gamble *et al.*[24], respectively, after extraction according to Bligh and Dyer[25]. The same extraction method was applied for hepatic lipids, after which commercially available kits were used for the determination of unesterified and total cholesterol (Wako, Neuss, Germany), and for triglycerides (Roche, Mannheim, Germany). Pooled plasma samples from all animals of one group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) as described previously[26].

### *RNA isolation and PCR procedures.*

Total RNA was extracted from frozen tissues with TriReagent (Sigma, St. Louis, MO, USA) and quantified photometrically. cDNA synthesis was performed using recombinant M-MLV reverse transcriptase (10 U/µl), the appropriate buffer, dNTPs (500 µM), random nonamers (1 µM), RNAse inhibitor (2 U/µl; all from Sigma) and total RNA (50 ng/µl). The reaction mix was incubated for 10 minutes at 25°C for primer annealing, 60 minutes at 37°C for synthesis and 5 minutes at 94°C to denature the RT enzyme. Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector as previously described[27]. Primers were obtained from Invitrogen (Carlsbad, USA). Fluorogenic probes, labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes used in these studies have been described elsewhere (Sterol regulatory element binding protein (*Srebp)1a, Srebp1c, Srebp2, Lxra,* scavenger receptor *(Srb)1,* acyl-coenzymeA: cholesterol acyltransferase *(Acat)1, Acat2,* 3-hydroxy-3-methylglutaryl-CoA reductase *(Hmgr),* cytochrome P-450 *(Cyp)7a1, Cyp27, Abca1, Abcg5, Abcg8* multidrug resistance protein*,( Mdr)2,* BS export protein *(Bsep),* and *18S rRNA*[27]; *beta actin* and LDL-receptor (*Ldlr)*[28]; *Abcg1*, *Abcg2*[29]; Niemann-Pick disease-1 (Npc1)-like 1 (*Npc1l1)* and *36b4*[30]; and *Mrp2* (*Abcc2*)[31]; with the exception of *Cyclophilin* (XM_356256; forward CAGATCGAGGGATCGATTCAG, reverse TCAC-CACTTGACACCCTCATTC, probe CTCCTCCACATTGGAGACAAGAGATGCA). All data of the Abcg5 experiment were subsequently normalized to the median of *beta-actin*, *36b4*, *18S rRNA*, and *cyclophilin* as described by Vandesompele *et al.*[32]. In the Lxrα experiments, *beta-actin* alone was used for normalization.

### *Statistics*

Statistical analyses were performed using SPSS 10.1 for Windows (SPSS Inc., Chicago, USA). Differences between genotypes were evaluated using the Mann-Whitney-U-test. A p-value smaller than 0.05 was considered statistically significant.

# RESULTS

### *Plasma and hepatic lipid composition in Abcg5$^{-/-}$ mice.*

It has been previously reported[6] that *Abcg5$^{-/-}$* mice have elevated plasma triglyceride levels compared to wild-type mice, whereas plasma cholesterol concentrations (measured by gas chromatography) are decreased. To establish the distribution of plasma sterols across the various lipoprotein classes, plasma samples were subjected to FPLC separation (Fig. 1). Total sterol (cholesterol + plant sterols) distribution was virtually identical in the two genotypes and almost exclusively present in the HDL-sized fractions.

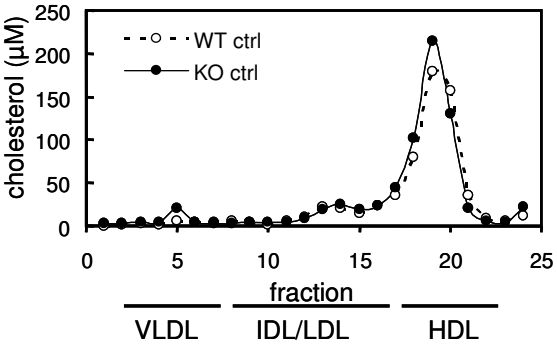


**Figure 1:** Distribution of sterols in plasma lipoprotein fractions of wild-type and *Abcg5$^{-/-}$* mice. Blood was collected via cardiac puncture and pooled before FPLC analysis. Analysis was performed as described in MATERIALS AND METHODS.

**TABLE 1.** Liver parameters of male *Abcg5$^{+/+}$*, *Abcg5$^{+/-}$* and *Abcg5$^{-/-}$* mice on chow diet

| | *Abcg5$^{+/+}$* | *Abcg5$^{+/-}$* | *Abcg5$^{-/-}$* |
|---|---|---|---|
| Ratio liver weight/body weight | 0.049 ± 0.002 | 0.049 ± 0.004 | 0.058 ± 0.002[a] |
| Total sterols (nmol/mg) | 5.14 ± 0.64 | 5.52 ± 0.72 | 5.24 ± 0.43 |
| Sterol ester (nmol/mg) | 0.77 ± 0.29 | 0.79 ± 0.50 | 0.43 ± 0.19[a] |
| Unesterified sterols (nmol/mg) | 4.37 ± 0.53 | 4.73 ± 0.26 | 4.80 ± 0.44 |
| Triglycerides (nmol/mg) | 19.2 ± 15.4 | 11.7 ± 9.8 | 8.9 ± 3.5 |
| Phospholipids (nmol/mg) | 34.8 ± 1.4 | 36.0 ± 0.4 | 36.8 ± 2.1 |

Male *Abcg5$^{-/-}$*, *Abcg5$^{+/-}$* and littermate control mice, 5-7 months old, were fed standard laboratory chow. Mice were anesthetized with isoflurane and killed by cardiac puncture. Livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses. Lipids were extracted and analysed as described in Materials and Methods. No distinction was made between cholesterol and other sterols. [a] indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$). n=5-7 per group.

As previously reported[6], liver weight was slightly increased in *Abcg5$^{-/-}$* mice compared to wild-type littermates (Table 1). This was not due to steatosis, because triglyceride concentrations were not increased in the knock-out mice. The concentrations of phospholipids as well as that of total sterols and unesterified sterols were almost identical between all groups. Only sterol ester concentrations in *Abcg5$^{-/-}$* mice were reduced by 45 %

compared to wild-type and heterozygotes. It should be noted that sterol concentrations were measured enzymatically and consisted of both cholesterol and plant sterols; it has been previously shown[6] that plant sterols comprise up to 42 % of total sterols in livers of *Abcg5*$^{-/-}$ mice while plant sterols are present in low amounts in livers of wild-type mice.

**TABLE 2.** Hepatic mRNA expression levels in male *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice on chow diet measured by real-time RT-PCR

| mRNA | *Abcg5*$^{+/+}$ | *Abcg5*$^{+/-}$ | *Abcg5*$^{-/-}$ |
|---|---|---|---|
| *Srebp1a* | 1.00 ± 0.11 | 1.11 ± 0.11 | 1.06 ± 0.12 |
| *Srebp1c* | 1.00 ± 0.41 | 1.08 ± 0.29 | 0.95 ± 0.42 |
| *Srebp2* | 1.00 ± 0.14 | 1.08 ± 0.35 | 0.78 ± 0.17 |
| *Lxra* | 1.00 ± 0.08 | 0.99 ± 0.12 | 1.05 ± 0.08 |
| *Ldlr* | 1.00 ± 0.11 | 1.18 ± 0.24 | 0.78 ± 0.28 |
| *Srb1* | 1.00 ± 0.20 | 1.06 ± 0.19 | 0.98 ± 0.10 |
| *Hmgcr* | 1.00 ± 0.35 | 1.36 ± 0.56 | 0.50 ± 0.23[a] |
| *Acat1* | 1.00 ± 0.11 | 0.94 ± 0.25 | 1.19 ± 0.20 |
| *Acat2* | 1.00 ± 0.18 | 1.24 ± 0.28 | 0.89 ± 0.32 |
| *Cyp7a1* | 1.00 ± 0.59 | 0.85 ± 0.66 | 1.82 ± 0.59[a] |
| *Cyp27* | 1.00 ± 0.13 | 1.24 ± 0.14 | 1.22 ± 0.22 |
| *Abcg8* | 1.00 ± 0.19 | 1.41± 0.44 | 1.26 ± 0.37 |
| *Abca1* | 1.00 ± 0.12 | 1.01 ± 0.09 | 1.24 ± 0.11[a] |
| *Abcg1* | 1.00 ± 0.36 | 0.98 ± 0.21 | 1.04 ± 0.13 |
| *Abcg2* | 1.00 ± 0.29 | 0.96 ± 0.19 | 0.98 ± 0.20 |
| *Npc1* | 1.00 ± 0.33 | 0.91 ± 0.43 | 1.19 ± 0.20 |
| *Npc1l1* | 1.00 ± 0.13 | 0.97 ± 0.60 | 1.41 ± 0.26[a] |
| *Bsep* | 1.00 ± 0.18 | 1.00 ± 0.13 | 0.99 ± 0.05 |
| *Mrp2* | 1.00 ± 0.32 | 1.37 ± 0.43 | 1.26 ± 0.36 |

Male *Abcg5*$^{-/-}$, *Abcg5*$^{+/-}$ and littermate control mice, 5-7 months old, were fed standard laboratory chow. Mice were anesthetized with isoflurane and killed by cardiac puncture. Liver pieces were snap frozen in liquid nitrogen and stored at -80°C. mRNA extraction, cDNA synthesis and TaqMan PCR analysis was done as described in Materials and Methods. All data were normalized to the median of *beta-actin*, *36b4*, *18S rRNA*, and *cyclophilin* as described by Vandesompele *et al.*[32]. n=5-8 per group. [a] indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$).

### *Hepatic gene expression.*

In male mice, hepatic gene expression was determined by real-time RT-PCR (Table 2). The mRNA levels of regulatory genes (*Srebp1a*, *1c*, *2*; *Lxra*) were identical in all groups. Expression of hepatic cholesterol uptake systems, i.e., *Ldlr* and *Srb1*, also did not differ between groups. Western blot analysis of isolated plasma membranes confirmed that also Sr-b1 protein levels were not different between genotypes (data not shown). On the other hand, expression of cholesterol metabolism genes in liver was different in *Abcg5*$^{-/-}$ mice compared to wild-type and *Abcg5*$^{+/-}$ mice: the expression of *Hmgcr*, rate-controlling for cholesterol synthesis was reduced by 50 % in *Abcg5*$^{-/-}$ mice. Expression of *Cyp7a1*,

encoding the enzyme responsible for the majority of bile salt synthesis, was upregulated by 82 % in *Abcg5*$^{-/-}$ mice, whereas that of *Cyp27* was unchanged. Expression levels of *Acat1* and *Acat2*, involved in cholesteryl ester formation, were not impaired in *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice compared to wild-type controls.

Gene expression of a wide spectrum of transporters potentially involved in cholesterol transport was screened: only the expression of *Abca1* (+ 24 %) and *Npc1l1* (+ 41 %) did show significant increases in *Abcg5*$^{-/-}$ mice compared to their wild-type littermates. Expression of *Abcg1*, *Abcg2*, *Abcg8*, *Npc1*, *Mrp2* and *Bsep* was identical in all three genotypes. As anticipated, *Abcg5* mRNA was virtually absent (5 % mRNA remaining compared to wild-type) in *Abcg5*$^{-/-}$ mice (Figure 2A). Surprisingly, *Abcg5* expression in heterozygous mice was 76 % of wild-type values. No effect on the expression of the phospholipid flippase *Mdr2* (*Abcb4*) was noticed (Figure 2B).

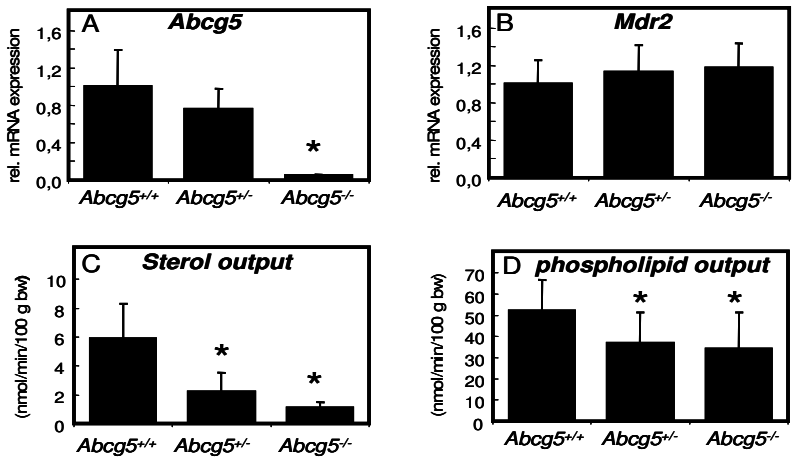


**Figure 2:** Hepatic *Abcg5* and *Mdr2* mRNA expression levels (above) and hepatobiliary sterol and phospholipid output rates (below) of *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice. **A** and **B**: Liver pieces were snap frozen in liquid nitrogen. mRNA extraction, cDNA synthesis and TaqMan PCR analysis was done as described in Materials and Methods. All data were normalized to the median of *beta-actin*, *36b4*, *18S rRNA*, and *cyclophilin* as described by Vandesompele *et al.*[32]. * indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$). **C** and **D**: Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder for 15 minutes. Biliary phospholipid and sterol concentrations were determined enzymatically as described previously[21]; no distinction was made between cholesterol and plant sterols. * indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$).

### *Biliary sterol and phospholipid secretion rates are decreased in Abcg5*$^{-/-}$ *mice.*

*Abcg8*$^{-/-}$ mice have been reported to have decreased hepatobiliary cholesterol and phospholipid secretion rates[5], while for *Abcg5*$^{-/-}$ mice only gallbladder concentration data are available[6]. Therefore, hepatic bile was collected from *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice for 15 minutes immediately after creation of a gallbladder fistula to determine basal biliary lipid output rates. Abcg5 knockout mice presented with a significantly increased bile flow compared to heterozygotes and wild-type littermates (8.9 ± 2.3 in *Abcg5*$^{-/-}$ mice *vs.* 6.6 ± 1.5

and 5.0 ±2.9 µl/min/100 g body weight in *Abcg5*$^{+/+}$ and *Abcg5*$^{+/-}$ mice, respectively). Hepatobiliary sterol (Fig. 2C) and phospholipid excretion rates (Fig. 2D) were significantly decreased in both heterozygous and homozygous knockout mice compared to wild-type controls. Bile salt output rates were unaffected, i.e., 325 ± 121, 309 ± 198 and 332 ± 164 nmol/min/100 g body weight in *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice, respectively. Moreover, gas-chromatographic analysis revealed that biliary bile salt composition was not significantly different between *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice (data not shown).

### *Biliary lipid excretion increases upon infusion with TUDCA in Abcg5*$^{-/-}$*, Abcg5*$^{+/-}$ *and wild-type mice.*

Systemic infusion of hydrophilic bile salts increases bile flow and facilitates hepatobiliary lipid secretion in wild-type mice[33]. To investigate whether this forced flow could restore impaired phospholipid- and sterol secretion in *Abcg5*$^{-/-}$ mice, we infused *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice with increasing concentrations of tauroursodeoxycholate (TUDCA). Bile flow was increased in *Abcg5*$^{-/-}$ mice compared to wild-type and heterozygote mice already under basal conditions. This effect was even more pronounced at higher infusion rates (data not shown).

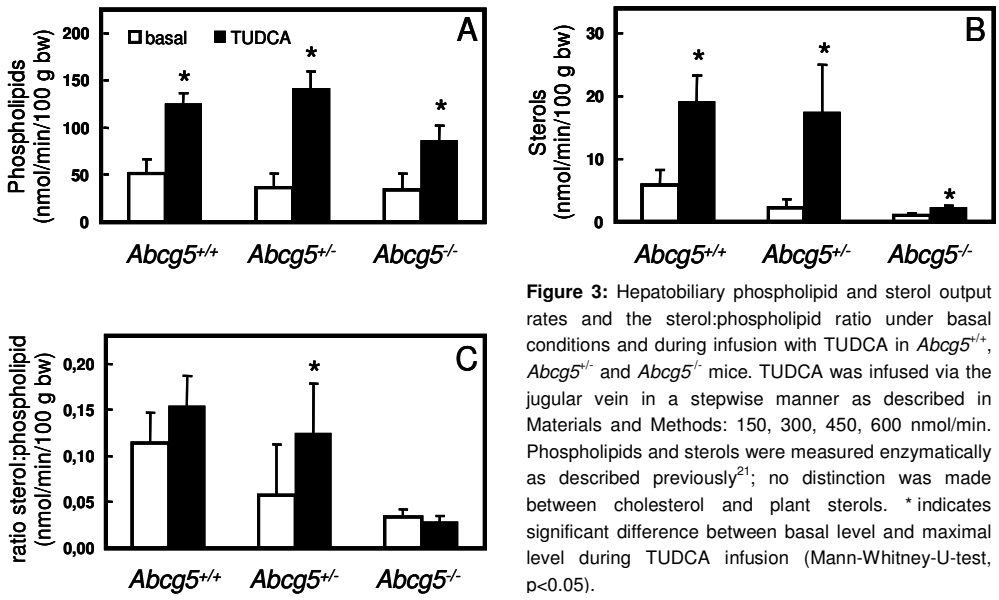


**Figure 3:** Hepatobiliary phospholipid and sterol output rates and the sterol:phospholipid ratio under basal conditions and during infusion with TUDCA in *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice. TUDCA was infused via the jugular vein in a stepwise manner as described in Materials and Methods: 150, 300, 450, 600 nmol/min. Phospholipids and sterols were measured enzymatically as described previously[21]; no distinction was made between cholesterol and plant sterols. * indicates significant difference between basal level and maximal level during TUDCA infusion (Mann-Whitney-U-test, $p<0.05$).

As depicted in Figure 3, phospholipid excretion increased upon TUDCA infusion in all genotypes (+140 %, +280 % and +150 % in *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice, respectively, during infusion of the highest dose of TUDCA). In parallel, hepatobiliary sterol excretion also increased in all strains (+230 %, + 700 %, and + 120 % *Abcg5*$^{+/+}$, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice, respectively). Noteworthy, the initially low sterol excretion rate in *Abcg5*$^{+/-}$ mice recovered upon infusion of TUDCA to reach wild-type-levels. As a consequence, the

sterol:phospholipid-ratios in *Abcg5*$^{+/-}$ mice and in *Abcg5*$^{-/-}$ mice were significantly lower than in wild-type mice on the basal level, but upon infusion with TUDCA the ratios normalized in *Abcg5*$^{+/-}$ mice but not in *Abcg5*$^{-/-}$ mice.

### *Biliary lipid excretion increases upon infusion with TDCA in Abcg5*$^{-/-}$ *and wild-type mice.*

To determine whether a more hydrophobic bile salt would restore hepatobiliary cholesterol excretion more effectively than TUDCA in *Abcg5*$^{-/-}$ mice, *Abcg5*$^{-/-}$ mice and littermate controls were infused with increasing amounts of the hydrophobic bile salt taurodeoxycholate (TDCA, Figure 4). At infusion rates of up to 75 nmol/min, bile flow and hepatobiliary bile salt output were indistinguishable between the two groups. At 100 nmol TDCA/min, the highest infusion rate tested, bile of the Abcg5 knockout mice turned red and bile flow dramatically decreased.

Both phospholipid excretion curves and sterol excretion curves were lower in the *Abcg5*$^{-/-}$ mice compared to wild-type controls. However, both phospholipid (3.5-times) and sterol (2.4-times) excretion increased in *Abcg5*$^{-/-}$ mice upon infusion with TDCA (at the maximal capacity of hepatobiliary lipid secretion upon an infusion rate of 75 nmol/min).This increase was not statistically different from that in wild-type mice (2.1-times for phospholipids and 3.5-times for sterols).

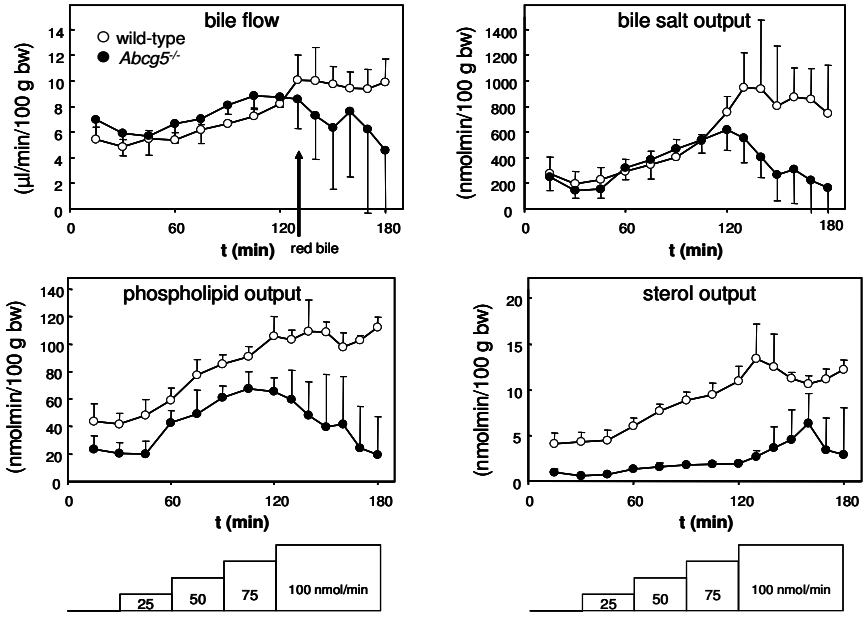


**Figure 4:** Bile flow and hepatobiliary bile salt, phospholipid and sterol output rates during infusion with increasing amounts of TDCA in *Abcg5*$^{-/-}$ and wild-type mice. TDCA was infused via the jugular vein at rates of 25, 50, 75 and 100 nmol/min. Measurements were done as described in MATERIALS AND METHODS. The arrow points at the appearance of red-colored bile in *Abcg5*$^{-/-}$ mice

### *The Abcg5-independent sterol secretion is not induced upon LXR activation.*

LXR is involved in the regulation of cholesterol homeostasis at various levels. To unravel if the remaining sterol excretion capacity in *Abcg5*$^{-/-}$ mice is LXR-dependent, wild-type and *Abcg5*$^{-/-}$ mice were fed the LXR agonist T0901317 for two weeks. Similar to results obtained previously[28], LXR activation led to the presence of large, triglyceride-rich HDL particles in *Abcg5*$^{-/-}$ mice (data not shown). As also reported previously[27], treatment with T0901317 yielded an increased hepatobiliary sterol secretion (+210 %) and a reduced phospholipid secretion (-35 %), resulting in a relative sterol hypersecretion in wild-type mice. In *Abcg5*$^{-/-}$ mice, however, no increase in sterol secretion was observed. In contrast, a slight drop of the already low sterol secretion rate occurred (from 1.1 to 0.7 nmol/min/100 g BW).

Upon infusion with TUDCA, phospholipid secretion rates more than doubled in all groups. The maximal excretory rate for sterols went up 4 times in wild-type mice treated with the LXR agonist compared to non-treated wild-type mice. However, in *Abcg5*$^{-/-}$ mice no difference was observed between LXR-treated and non-treated mice. Figure 5 visualizes the effect of LXR activation in *Abcg5*$^{-/-}$ mice and wild-type littermates. The sterol:bile salt ratio was clearly increased in wild-type mice upon treatment with T0901317, whereas the relationships between bile salt and cholesterol output in untreated or T0901317-treated *Abcg5*$^{-/-}$ mice were similar.

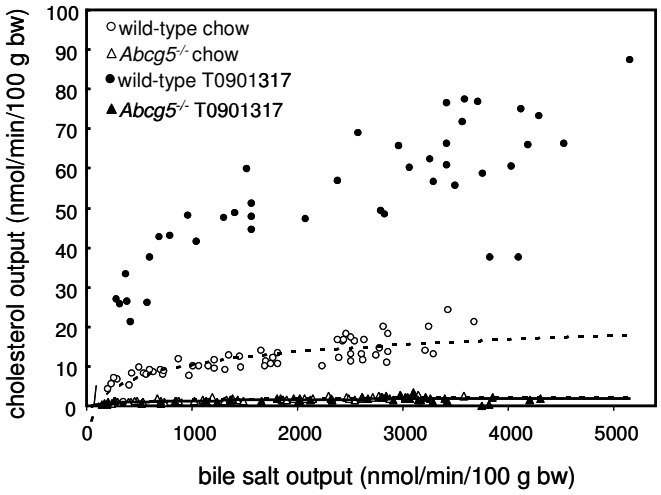


**Figure 5:** The effect of LXR activation in *Abcg5*$^{-/-}$ mice and wild-type littermates during TUDCA infusion on hepatobiliary cholesterol output. TUDCA was infused via the jugular vein in a stepwise manner as described in MATERIALS AND METHODS: 150, 300, 450, 600 nmol/min. Sterols were measured enzymatically as described previously[21]; no distinction was made between cholesterol and plant sterols.

### *Cholesterol feeding increases hepatobiliary cholesterol excretion in wild-type and Lxra*$^{-/-}$ *mice.*

To test whether the reported increase in hepatobiliary cholesterol excretion upon cholesterol feeding is LXR- (and Abcg5/Abcg8) dependent, *Lxra*$^{-/-}$, *Lxra*$^{+/-}$ and wild-type control mice were fed a diet containing 1 % cholesterol to increase delivery of cholesterol to the liver. Hepatic sterol(ester) contents increased to similar levels in wild-type and heterozygote mice, but much stronger in the *Lxra*$^{-/-}$ mice. As anticipated[15], hepatic expression of *Abcg5* and *Abcg8* was increased in control mice and heterozygotes on high-cholesterol diet (Figure 6).

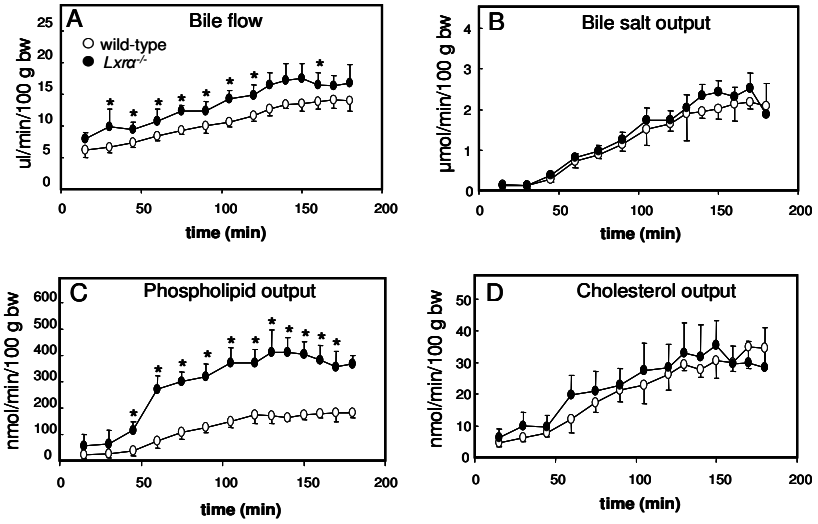


**Figure 6:** Hepatic Abcg5 and Abcg8 mRNA expression levels (right) and hepatobiliary cholesterol and phospholipid output rates (left) of *Lxra*$^{+/+}$, *Lxra*$^{+/-}$ and *Lxra*$^{-/-}$ mice fed chow (open bars) or high-cholesterol diet (filled bars). **A** and **B**: Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder for 30 minutes. Biliary sterol and phospholipid concentrations were determined as described in Materials and Methods. * indicates significant difference between chow-fed and high-cholesterol groups (Mann-Whitney-U-test, $p<0.05$). **C** and **D**: Liver pieces were snap frozen in liquid nitrogen. mRNA extraction, cDNA synthesis and TaqMan PCR analysis was done as described in Materials and Methods. All data were normalized to *beta-actin*. * indicates significant difference between chow-fed and high-cholesterol groups (Mann-Whitney-U-test, $p<0.05$).

In *Lxra*$^{-/-}$ mice on high cholesterol diet, Abcg5 and Abcg8 expression did not differ from that in chow-fed *Lxra*$^{-/-}$ mice. Bile flow, biliary bile salt and phospholipid excretion rates did not differ between the groups. Surprisingly, hepatobiliary cholesterol excretion was increased in all mice on high cholesterol diet, regardless of genotype (Figure 6).

### *Hepatobiliary cholesterol excretion in Lxra$^{-/-}$ mice is correlated with increased phospholipid secretion upon infusion of TUDCA.*

In principle, it could be speculated that basal expression levels of *Abcg5* and *Abcg8* are sufficiently high to allow for the increased hepatobiliary cholesterol transport in the absence of LXR. To test this hypothesis, we infused *Lxra*$^{-/-}$ and wild-type mice fed high-cholesterol diet with increasing amounts of TUDCA to determine the maximal secretory rates for biliary lipids. As depicted in Figure 7, bile flow was slightly higher in *Lxra*$^{-/-}$ mice than in littermate controls, whereas bile salt output was identical. Unexpectedly, hepatobiliary cholesterol secretion rates were identical in *Lxra*$^{-/-}$ and wild-type mice even at the highest TUDCA infusion rate applied. Moreover, the hepatobiliary excretion of phospholipids was up to 200% increased in *Lxra*$^{-/-}$ mice compared to wild-type littermates. As a result, the cholesterol/phospholipid ratio indicated a relative hypersecretion in wild-type (ratio 0.176) compared to *Lxra*$^{-/-}$ mice (ratio 0.088).

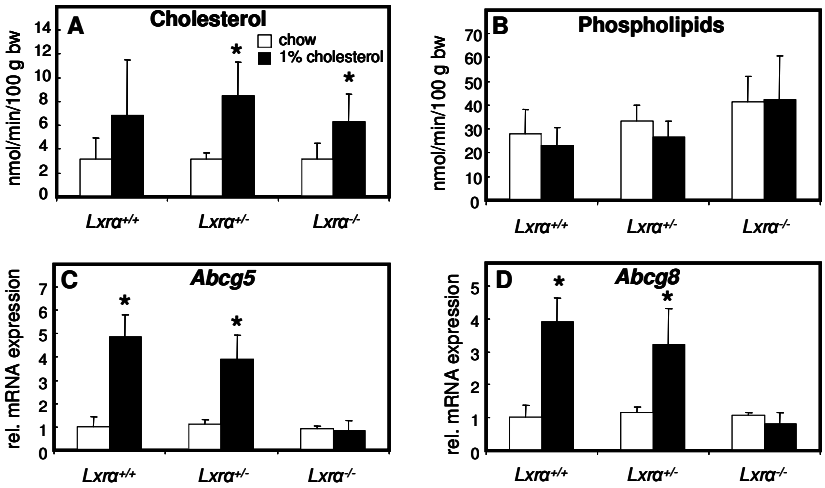


**Figure 7:** Bile flow and hepatobiliary bile salt, phospholipid and sterol output rates during infusion with increasing amounts of TUDCA in *Lxra*$^{-/-}$ (filled circles) and wild-type mice (open circles) fed high-cholesterol diet. Male *Lxra*$^{-/-}$ mice and their wild-type littermates received either standard mouse chow or chow diet containing 1 % cholesterol for two weeks. TUDCA was infused via the jugular vein at rates of 25, 50, 75 and 100 nmol/min. Measurements were done as described in MATERIALS AND METHODS.

Expression of *Mdr2/Abcb4*, the gene coding for the canalicular phospholipid flippase Mdr2 P-glycoprotein, was measured in liver tissues of *Lxra*$^{-/-}$, *Lxra*$^{+/-}$ and wild-type mice on chow and high-cholesterol diet. All groups but *Lxra*$^{-/-}$ mice on high-cholesterol diet showed similar expression levels; in *Lxra*$^{-/-}$ mice, however, *Mdr2* expression was reduced to 60 % of wild-type level (data not shown).

## DISCUSSION

The mechanism by which cholesterol molecules are excreted from the hepatocyte into the bile still represents an unsolved problem in lipid biochemistry. The discovery of the ABC-half transporters Abcg5 and Abcg8 as important players herein[1,2] has established the involvement of these transporter proteins, but mechanistic issues concerning the actual excretion process have remained in the dark. Knocking out the genes encoding Abcg5, Abcg8, or both in mice dramatically reduces biliary cholesterol concentrations[5,6,17], which was taken to indicate that Abcg5 and Abcg8 function as a heterodimer in the excretion process. The fact that heterozygous knock-out mice and rodents in which *Abcg5/Abcg8* expression is modulated[6,11,14-16] show a clear phenotype for biliary cholesterol excretion underscores the important role of the Abcg5/Abcg8 heterodimer in control of the secretion process. Currently, two major models have been proposed concerning the mode of action of the Abcg5/Abcg8 heterodimer in hepatobiliary cholesterol excretion. The first model, basically proposed by Wittenburg and Carey[34], postulates that the Abcg5/Abcg8 heterodimer translocates ("flops") cholesterol from the inner leaflet of the canalicular membrane to the

outer one and thereby provides a continuous supply of cholesterol for (bile salt-facilitated) excretion. An alternative model by Small[35] proposes that cholesterol reaches the outer leaflet by diffusion. There, the Abcg5/Abcg8 heterodimer would "lift" cholesterol from its membraneous environment to be more easily available for micellization by bile salts present in the lumen of the canaliculus. In this study we wanted to address the extent of rate-control exerted by the Abcg5/Abcg8 heterodimer in hepatobiliary (chole)sterol secretion under conditions of (maximal) induction of this process.

First, it was demonstrated that sterols present in plasma of *Abcg5*$^{-/-}$ mice, like in wild-type mice, are almost exclusively present in HDL-sized fractions. Furthermore, hepatic mRNA and protein expression of the HDL-receptor Sr-b1 was not affected by *Abcg5*-deficiency. This excludes differences in "delivery" of plasma cholesterol to the liver as a cause for the observed differences in biliary cholesterol secretion. However, it cannot be ruled out that enlargement of HDL particles upon LXR activation[28,36] leads to a differential processing by livers from wild-type and knock-out mice.

In a very recent study[16], Yu and colleagues have elegantly demonstrated that gallbladder cholesterol concentrations correlate with *Abcg5/Abcg8* expression levels in transgenic mice containing 1, 10, or 16 copies of *Abcg5/Abcg8*. Likewise, Kosters *et al.*[13] have demonstrated a high correlation between "normalized" biliary cholesterol secretion and hepatic *Abcg5*/*Abcg8* mRNA expression when various mouse models were compared. In this study, we quantified hepatobiliary cholesterol excretion rates, rather than gallbladder concentrations, in wild-type, *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice. We have recently reported that cholesterol concentrations in the gallbladder of *Abcg5*$^{-/-}$ mice are reduced by 50 % compared to wild-type mice[6]. In this study, we found a much stronger reduction of biliary cholesterol output rates, as measured after cannulation of the gallbladder. The discrepancy between the low hepatic cholesterol output rates and relatively higher downstream concentrations in the gallbladder may be caused by processes in the gallbladder itself. It can be speculated that gallbladder epithelial cells are able to deliver cholesterol to the bile, particularly when detergent biliary bile salts are not shielded sufficiently by phospholipids and cholesterol.

Both hepatobiliary phospholipid and sterol secretion were impaired under basal, non-stimulated conditions in *Abcg5*$^{+/-}$ and *Abcg5*$^{-/-}$ mice. Hepatic expression of *Abcg5* was reduced by only 24 % in the heterozygotes, indicative for compensatory up-regulation of transcription of the remaining allele, whereas that of *Mdr2* (*Abcb4*) was not affected. The relatively small reduction of *Abcg5* expression in heterozygotes was associated with a reduction by approximately 60% of biliary sterol secretion. Therefore, it is reasonable to conclude that, under these conditions, *Abcg5* expression controls a large fraction of hepatobiliary sterol secretion and partly, either directly or indirectly, also influences phospholipid secretion. It is tempting to speculate that reduced phospholipid secretion associated with *Abcg5*-deficiency is a secondary phenomenon, most likely related to altered canalicular membrane structure or biliary micelle composition. It is well-established that, under physiological circumstances, biliary phospholipid and cholesterol concentrations are coupled to that of bile salts[37]. To exclude the possibility that changes in biliary bile salt composition contributed to the reduced phospholipid and cholesterol excretion rates, bile salt composition was determined. It is noteworthy that biliary bile salt composition was indistinguishable between the three genotypes, as was expression of the canalicular bile salt transporters *Bsep* (*Abcb11*) and *Mrp2* (*Abcc2*).

The high degree of control on biliary excretion was overcome when biliary bile salt secretion was stimulated by infusion of the hydrophilic bile salt TUDCA. Under these conditions, both sterol and phospholipid secretion rates approximated wild-type levels in the heterozygotes, resulting in a normalization of the sterol:phospholipid ratio in bile. This indicates that a high bile salt flux creates a situation in which biliary sterol and phospholipid excretion becomes less dependent of *Abcg5* expression levels. Under the same conditions, biliary sterol- and phospholipid secretion in *Abcg5*$^{-/-}$ mice did increase substantially, indicating that sterol secretion is inducible in the absence of Abcg5, but remained low when compared to *Abcg5*$^{+/+}$ and *Abcg5*$^{+/-}$ mice. It is tempting to speculate that the observed increase reflects an Abcg5/Abcg8-independent, but bile salt-dependent part of biliary cholesterol secretion that contributes approximately 20 % of maximal output under chow-fed conditions.

Infusion of the hydrophobic bile salt TDCA restored cholesterol secretion, but not phospholipid secretion, in mice lacking Mdr2 (*Abcb4*)[33]. TDCA infusions at low concentrations increased phospholipid and sterol secretion in *Abcg5*$^{-/-}$ mice at the same *relative* rate as observed in wild-type mice, however, at a much lower *absolute* level. At higher infusion rates of TDCA, bile of *Abcg5*$^{-/-}$ mice only turned red and the mice became cholestatic. It is likely that induction of cholestasis reflects detergent effects of intraluminal TDCA exerted on canalicular membranes in the absence of sufficient amounts of cholesterol in the outer leaflet of the canalicular membrane. This rapid induction of cholestasis by TDCA may be considered as support, but definitively not as proof, for a floppase mode of action of the Abcg5/Abcg8 heterodimer, since the liftase model would predict cholesterol to be present in similar or even higher amounts in the outer leaflet of the canalicular membrane in Abcg5$^{-/-}$ mice compared to wild-type mice. It should be realized, however, that the exact mechanisms of TDCA-induced cholestasis is not yet known.

In a series of classical papers[38-41], Yousef and colleagues studied the effects of different bile salts on bile flow and biliary lipid composition in rats. Upon infusion of hydrophobic bile salts, typically phospholipid secretion declined first, *followed* by decreases in bile flow, bile salt output and cholesterol output. Concomitantly with the decline in phospholipid output, phospholipid composition changed from mainly phosphatidylcholine to more phosphatidylethanolamine and sphingomyelins, which was attributed to partial solubilization of the canalicular membrane[41]. Based on these results, these authors concluded that insufficient supply of phosphatidylcholine to the canalicular plasma membrane was the cause of bile salt-induced cholestasis. Our data from TDCA-infused *Abcg5*$^{-/-}$ mice, however, differ in the kinetics of the process reported by Yousef *et al.*: in *Abcg5*$^{-/-}$ mice, the maximal secretory rate for phospholipids and bile salts as well as the maximal bile flow rate were reached *earlier* than that of sterols. This would indicate that in the outer leaflet of the canalicular membrane, sterols were present - even in the absence of Abcg5 - which could be "dissolved" by hydrophobic micelles. However, the appearance of red bile suggests that the late increase in sterol excretion in the *Abcg5*$^{-/-}$ mice may also be caused by hepatic micro-bleedings which could theoretically provide erythrocyte membranes as a source for the sterols measured in bile. In any case, our data demonstrate that the absence of a functional Abcg5/Abcg8 heterodimer renders the mouse susceptible to bile salt-induced cholestasis.

Treatment with the LXR agonist T0901317 has been demonstrated to increase expression of *Abcg5*, *Abcg8*, *Abca1* and other genes involved in cholesterol transport[11,15].

LXR activation dramatically increased sterol excretion in wild-type mice under basal conditions and upon infusion with TUDCA. However, no additional effect of LXR activation was observed in *Abcg5*$^{-/-}$ mice. Thus, the remaining sterol secretion in *Abcg5*-deficient mice is independent from LXR-activated systems. Interestingly, Sehayek and colleagues[42] reported that Abcg5/Abcg8-independent loci regulate plasma plant sterol levels in mice. This further supports the hypothesis that other mechanisms than Abcg5/Abcg8-mediated transport exist to regulate sterol homeostasis.

To further substantiate the existence of Abcg5-independent cholesterol secretion, we examined a model in which the expression of this transporter remains unchanged upon loading of the liver with dietary cholesterol, *i.e.*, the *Lxra*$^{-/-}$ mouse on a high cholesterol diet. Wild-type mice show a strong up-regulation of hepatic *Abcg5*/*Abcg8* expression when challenged with a high cholesterol diet[15]. This response is mediated via LXR and, consequently, abolished in *Lxra*$^{-/-}$ mice. Nevertheless, both wild-type and *Lxra*$^{-/-}$ mice showed a significant increase in hepatobiliary cholesterol excretion, independent of the *Abcg5*/*Abcg*8 expression level. This could either mean that the Abcg5/Abcg8 heterodimer is not rate-controlling under these conditions or that other routes compensate for this system under this particular stress. Increased hepatobiliary phospholipid excretion in *Lxra*$^{-/-}$ mice without induction of *Mdr2/Abcb4* expression might indicate that enhanced micelle formation at the outer leaflet of the canalicular membrane could play a role under this particular circumstances.

Across various mouse models, a strong correlation exists between biliary cholesterol excretion and hepatic *Abcg5/g8* expression[13], with the notable exception of the diosgenin-treated mouse. Diosgenin is a plant sterol-like compound known to induce hypersecretion of cholesterol into bile[43], possibly dependent on PXR activation, but independent of the expression of Abcg5/g8[18]. In contrast to the mouse data published by Kosters *et al.*[13], in human liver transplantation patients no relationship between (normalized) biliary cholesterol excretion and ABCG5 and ABCG8 expression was found[19], indicating at least relatively large contributions of Abcg5/Abcg8-independent cholesterol excretion in this specific patient population. In mice, absence of LXRα in combination with a high dietary cholesterol intake seems to add another model in which Abcg5/Abcg8 expression does not correlate with cholesterol excretion rates. We, therefore, favor the hypothesis that (an) LXR- and Abcg5/Abcg8-independent route(s) of biliary cholesterol secretion might come into play in specific situations.

Taken together, our studies support the notion that Abcg5/Abcg8 has rate-controlling function for the majority of hepatobiliary cholesterol transport in mice under "basal" conditions and that it may function as a floppase. However, a considerable fraction of cholesterol may reach the bile via an Abcg5/Abcg8-independent route, particularly when the secretory process is stimulated by "classical" approaches, i.e., bile salt infusion or cholesterol feeding. Thus, in other words, changes in hepatic *Abcg5*/*Abcg8* expression alone do not always predict changes in the actual metabolic flux of interest, i.e., the hepatobiliary cholesterol secretion rate.

## ACKNOWLEDGEMENTS

We thank Juul F.W. Baller and Renze Boverhof for excellent technical assistance and Dr. A.K. Groen for helpful discussions. This study was supported by grant 912-02-063 from the Netherlands Organization for Scientific Research (NWO). Torsten Plösch is the recipient of a Dr. Dekker fellowship from the Dutch Heart Foundation (2004T048). Parts of this study have been presented at the annual meeting of the AASLD (Boston, 2004) and published in abstract form.

## REFERENCES

1. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R, Hobbs HH. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 2000;290:1771-1775.
2. Lee MH, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, Kojima H, Allikmets R, Sakuma N, Pegoraro R, Srivastava AK, Salen G, Dean M, Patel SB. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat Genet* 2001;27:79-83.
3. Bjorkhem I, Boberg KM, Leitersdorf E. Inborn errors in bile acid biosynthesis and storage of sterols other than cholesterol. In: Scriver C, Beaudet A, Sly W, Valle D, eds. *The Metabolic and Molecular Bases of Inherited Disease*. New York: McGraw-Hill; 2001:2961-2988.
4. Graf GA, Yu L, Li WP, Gerard R, Tuma PL, Cohen JC, Hobbs HH. ABCG5 and ABCG8 Are Obligate Heterodimers for Protein Trafficking and Biliary Cholesterol Excretion. *J Biol Chem* 2003;278:48275-48282.
5. Klett EL, Lu K, Kosters A, Vink E, Lee MH, Altenburg M, Shefer S, Batta AK, Yu H, Chen J, Klein R, Looije N, Oude-Elferink R, Groen AK, Maeda N, Salen G, Patel SB. A mouse model of sitosterolemia: absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol. *BMC Med* 2004;2:5.
6. Plösch T, Bloks VW, Terasawa Y, Berdy S, Siegler K, Van Der Sluijs F, Kema IP, Groen AK, Shan B, Kuipers F, Schwarz M, Schwartz M. Sitosterolemia in ABC-transporter G5-deficient mice is aggravated on activation of the liver-X receptor. *Gastroenterology* 2004;126:290-300.
7. Heinemann T, Axtmann G, Von Bergmann K. Comparison of intestinal absorption of cholesterol with different plant sterols in man. *Eur J Clin Invest* 1993;23:827-831.
8. Salen G, AHRENS EH, Jr., Grundy SM. Metabolism of beta-sitosterol in man. *J Clin Invest* 1970;49:952-967.
9. Igel M, Giesa U, Lutjohann D, Von Bergmann K. Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice. *J Lipid Res* 2003;44:533-538.
10. Freeman LA, Kennedy A, Wu J, Bark S, Remaley AT, Santamarina-Fojo S, Brewer HB, Jr. The orphan nuclear receptor LRH-1 activates the ABCG5/ABCG8 intergenic promoter. *J Lipid Res* 2004;45:1197-1206.
11. Yu L, York J, Von Bergmann K, Lutjohann D, Cohen JC, Hobbs HH. Stimulation of cholesterol excretion by the liver X receptor agonist requires ATP-binding cassette transporters G5 and G8. *J Biol Chem* 2003;278:15565-15570.
12. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 1996;383:728-731.
13. Kosters A, Frijters RJ, Schaap FG, Vink E, Plösch T, Ottenhoff R, Jirsa M, De Cuyper IM, Kuipers F, Groen AK. Relation between hepatic expression of ATP-binding cassette transporters G5 and G8 and biliary cholesterol secretion in mice. *J Hepatol* 2003;38:710-716.
14. Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC, Hobbs HH. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest* 2002;110:671-680.
15. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *J Biol Chem* 2002;277:18793-18800.
16. Yu L, Gupta S, Xu F, Liverman AD, Moschetta A, Mangelsdorf DJ, Repa JJ, Hobbs HH, Cohen JC. Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. *J Biol Chem* 2005;280:8742-8747.

17. Yu L, Hammer RE, Li-Hawkins J, Von Bergmann K, Lutjohann D, Cohen JC, Hobbs HH. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci U S A* 2002;99:16237-16242.
18. Kosters A, Frijters RJ, Kunne C, Vink E, Schneiders MS, Schaap FG, Nibbering CP, Patel SB, Groen AK. Diosgenin-induced biliary cholesterol secretion in mice requires Abcg8. *Hepatology* 2005;41:141-150.
19. Geuken E, Visser DS, Leuvenink HG, de Jong KP, Peeters PM, Slooff MJ, Kuipers F, Porte RJ. Hepatic expression of ABC transporters G5 and G8 does not correlate with biliary cholesterol secretion in liver transplant patients. *Hepatology* 2005;42:1166-1174.
20. Mashige F, Imai K, Osuga T. A simple and sensitive assay of total serum bile acids. *Clin Chim Acta* 1976;70:79-86.
21. Frijters CM, Ottenhoff R, Van Wijland MJ, van Nieuwkerk C, Groen AK, Oude Elferink RP. Influence of bile salts on hepatic mdr2 P-glycoprotein expression. *Adv Enzyme Regul* 1996;36:351-363.
22. Moghadasian MH, Frohlich JJ, Scudamore CH. Specificity of the commonly used enzymatic assay for plasma cholesterol determination. *J Clin Pathol* 2002;55:859-861.
23. Bottcher CFJ, van Gent CM, Pries C. A rapid and sensitive sub-micro-phosphorus determination. *Anal Chim Acta* 1961;24:203-204.
24. Gamble W, Vaughan M, Kruth HS, Avigan J. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J Lipid Res* 1978;19:1068-1070.
25. Bligh EG, Dyer WJ. Rapid Method of Total Lipid Extraction and Purification. *Can J Biochem Biophys* 1959;37:911-917.
26. Voshol PJ, Havinga R, Wolters H, Ottenhoff R, Princen HM, Oude Elferink RP, Groen AK, Kuipers F. Reduced plasma cholesterol and increased fecal sterol loss in multidrug resistance gene 2 P-glycoprotein-deficient mice. *Gastroenterology* 1998;114:1024-1034.
27. Plösch T, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, Groen AK, Kuipers F. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J Biol Chem* 2002;277:33870-33877.
28. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J Biol Chem* 2002;277:34182-34190.
29. Kruit JK, Plosch T, Havinga R, Boverhof R, Groot PH, Groen AK, Kuipers F. Increased fecal neutral sterol loss upon liver X receptor activation is independent of biliary sterol secretion in mice. *Gastroenterology* 2005;128:147-156.
30. van der Veen JN, Kruit JK, Havinga R, Baller JF, Chimini G, Lestavel S, Staels B, Groot PH, Groen AK, Kuipers F. Reduced cholesterol absorption upon PPARdelta activation coincides with decreased intestinal expression of NPC1L1. *J Lipid Res* 2005;46:526-534.
31. Kok T, Bloks VW, Wolters H, Havinga R, Jansen PL, Staels B, Kuipers F. Peroxisome proliferator-activated receptor alpha (PPARalpha)-mediated regulation of multidrug resistance 2 (Mdr2) expression and function in mice. *Biochem J* 2003;369:539-547.
32. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034.
33. Oude Elferink RP, Ottenhoff R, van Wijland M, Frijters CM, van Nieuwkerk C, Groen AK. Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P-glycoprotein. *J Lipid Res* 1996;37:1065-1075.
34. Wittenburg H, Carey MC. Biliary cholesterol secretion by the twinned sterol half-transporters ABCG5 and ABCG8. *J Clin Invest* 2002;110:605-609.
35. Small DM. Role of ABC transporters in secretion of cholesterol from liver into bile. *Proc Natl Acad Sci U S A* 2003;100:4-6.
36. Jiang XC, Beyer TP, Li Z, Liu J, Quan W, Schmidt RJ, Zhang Y, Bensch WR, Eacho PI, Cao G. Enlargement of high density lipoprotein in mice via liver X receptor activation requires apolipoprotein E and is abolished by cholesteryl ester transfer protein expression. *J Biol Chem* 2003;278:49072-49078.
37. Verkade HJ, Vonk RJ, Kuipers F. New insights into the mechanism of bile acid-induced biliary lipid secretion. *Hepatology* 1995;21:1174-1189.
38. Barnwell SG, Tuchweber B, Yousef IM. Biliary lipid secretion in the rat during infusion of increasing doses of unconjugated bile acids. *Biochim Biophys Acta* 1987;922:221-233.

39. Barnwell SG, Yousef IM, Tuchweber B. The effect of colchicine on the development of lithocholic acid-induced cholestasis. A study of the role of microtubules in intracellular cholesterol transport. *Biochem J* 1986;236:345-350.
40. Yousef IM, Barnwell SG, Tuchweber B, Weber A, Roy CC. Effect of complete sulfation of bile acids on bile formation in rats. *Hepatology* 1987;7:535-542.
41. Yousef IM, Barnwell S, Gratton F, Tuchweber B, Weber A, Roy CC. Liver cell membrane solubilization may control maximum secretory rate of cholic acid in the rat. *Am J Physiol* 1987;252:G84-G91.
42. Sehayek E, Duncan EM, Lutjohann D, Von Bergmann K, Ono JG, Batta AK, Salen G, Breslow JL. Loci on chromosomes 14 and 2, distinct from ABCG5/ABCG8, regulate plasma plant sterol levels in a C57BL/6J x CASA/Rk intercross. *Proc Natl Acad Sci U S A* 2002;99:16215-16219.
43. Cayen MN, Dvornik D. Effect of diosgenin on lipid metabolism in rats. *J Lipid Res* 1979;20:162-174.